# TAK1 as a potential link to regulate nutrient signaling through mTORC1 pathway

# PhD Synopsis


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| **Date of Registration** | 19 October 2021 |
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## 1. Background

TGF-β Activated kinase 1, a serine threonine kinase, was first discovered as a member of the MAPK kinase kinase (MAP3K) family, named as MAP3K7, and is activated by TGF-β1. Apart from TGF-β1, TAK1 can be activated by various other stimuli including lipopolysaccharides, pro-inflammatory cytokines like interleukin (IL)-1, tumor necrosis factor (TNF)-α and environmental stress. TGF-β activated kinase 1 has been associated with the regulation of various cellular processes which include embryonic development, differentiation, apoptosis, autophagy and cell survival. TAK1 in association with its binding partners TAB1, TAB2 and TAB3 sets out a composite regulation pattern thus engaging crucial signaling pathways like the C-Jun N-terminal kinase (JNK), p38 MAPK, and I-kappa B kinase complex (IKK) that are known to be involved in establishing cellular commitments for death and survival (Yoon Seok *et al*., 2014). With its emerging role in impacting mTORC1, and also being the upstream kinase of AMPK, it may coordinate the cellular energy homeostasis network. The kinase activity of TAK1 is regulated by a number of post- translational modifications including phosphorylation and ubiquitination. The deactivation of TAK-1 involves the action of certain phosphatases and de-ubiquitinases, which results in the deactivation of TAK-1.

MAPK kinase kinase family serine threonine kinase member: TAK-1, has emerged as a common link to relate cell survival and developmental mechanisms with various programs related to cellular death like autophagy and apoptosis. By engaging with the key sensor of energy homeostasis, AMPK and mTOR-the regulator of cell growth processes, the scope of TAK-1 as a key player in regulating cell survival and death dynamics has been vastly enhanced, which has accordingly paved a way for TAK1 to be a frontline candidate for therapeutic

interventions.

In addition to previously identified role of TAK1 in modulating the mTORC1 signaling pathways a central pathway of cellular growth and proliferation, we intend to evaluate the role of TAK1 in modulating mTORC1 activity.

Also, the mTORC1 pathway, which normally acts as an inhibitor of autophagy, is strangely active during cancer genesis. Because of a lack of knowledge, this contradiction has been dismissed as a context-dependent phenomenon that otherwise warrants further exploration. It is therefore critical to comprehend this context, which appears to maintain growth despite nutritional shortage through persistent mTORC1 activation. This background, together with our previous data, led us to propose an alternate mechanism of mTORC1 activation in nutrient-depleted settings, which must synchronise with autophagy dynamics. Accordingly, we intend to determine TAK1's role in cellular autophagy regulation and its link to mTORC1.

## 2. Aim

Understanding TAK1 as a potential link to regulate nutrient sensing via mTORC1 pathway.

## 3. Objectives

**1). To evaluate the Role of Tak1 in mTORC1 regulation.**

a) To examine the effect of TAK1 in governing mTORC1/S6K1 activity.

b) Interaction of TAK1 and S6K1

c) Mutational analysis of S6k1 binding domain.

**2). To investigate the effect of TAK1 on AMPK activation under the conditions of nutrient deficiency and/or nutrient availability.**

a) Construction of TAK1 knockout cell line.

b) Validation of TAK1 knockout cell line.

c) AMPK activation status in TAK1 knockout cell line

**3). To decipher the role of TAK1 in the regulation of cellular autophagy and its relationship with mTORC1.**

## 4. Review of literature

### 4.1. Tak1/ transforming growth factor–β activated kinase 1

TAK1 is a serine threonine kinase, which was first discovered as a member of mitogen activated kinase kinase kinase family, MAP3k and named as MAP3K7 (Wang *et al*., 2021). TAK1 was originally discovered in *Saccharomyces cerevisiae* pheromone induced MAPK pathway, by CDNA library screening and protein fragmentation complementation assay as Ste11p (mammalian homologue of MAPKKKs) (Yamaguchi *et al*., 1995). TAK1 was first identified as a member of MAPKKK family and was found to be activated in response to TGF-β or Bone morphogenic protein BMP-4 and mediate signal transduction related to this pathway (Yan-Ran XU *et al*., 2021). TAK1 was later on found to be activated in response to various stimuli, like interleukin-1 (IL-1), tumor necrosis factor-α and ligands of toll-like receptor (TLR). TGF- β can also be activated by lipopolysaccharides (LPS) and environmental stress (Shirakabi *et al*., 1997). TAK-1 is known to activate nuclear factor-κB (NF-κB) signaling pathway, mitogen activated protein kinase signaling (MAPK) pathway,C-Jun N-terminal kinase pathway (JNK) and I-Kappa B Kinase (IKK) pathways (Ninomiya-Tsuji.J *et al*., 1995, Shim JH *et al*., 2005). These pathways are the key regulators of various cellular processes which include cell development, differentiation, survival, apoptosis, autophagy, immune response, homeostasis and metabolism (Roh *et al*., 2014).

TAK1 is also implicated in the regulation of the non-canonical Wnt signaling and hence functions as a negative feedback control of canonical wnt signaling (Smit. L *et al*., 2004). Numerous evidences point towards the fact that TAK1 promotes the expression of Smad7 thus regulating Smad signaling induced by TGF-β (Dowdy SC *et al*., 2003). TAK1 also interacts with the MH2 domain of Smads which hinders the transactivation of R-Smads (Huffmann *et al*., 2001). In addition to the regulatory functions of Smads, there also exists a crosstalk among the Smads and the downstream effectors of TAK1 such as ATF2 and p38MAPK which regulates the TGF-β1 target gene expressions (Hanafusa. H *et al*., 1999, Sano. Y *et al* 1999, Abecassis. L et al., 2004). Added together these findings indicate that TAK1 might act to be a converging point among different signaling pathways triggered by various signals. TAK1 is involved in the regulation of differentiation, cell survival and inflammatory responses, and also in the process of apoptosis. depending upon the type of cell types and tissues, it may stimulate or impede apoptosis (Sorrentino *et al*., Sayama. K *et al*.,). TAK-1 has also been found to be involved in activating potent tumor suppressor proteins like pVHL (Tam. SH *et al*.,) and LKB1 (Xie. M *et al*.,).

TAK-1 activity is regulated by its association with various adaptor proteins known as TAK-1 binding proteins: TAB1, TAB2 and TAB3. TAK-1 activity is also regulated by various post-translation modifications like phosphorylation, dephosphorylation, ubiquitination and de-ubiquitination (Sabreena *et al*., 2019).

**4.2. Structure of TAK1**

The crystal structure of co-precipitated complex of un-phosphorylated TAK1–TAB1 (Ile31–Gln304:His468–Pro504) with adenosines has been determined. This structure of TAK1-TAB1 consists of a fold that is typical of protein kinases (Sicheri.F *et al*., 1998, Hanks. S. K *et al*., 1998). TAK1 has a large C-terminal lobe (constituted of residues 111 to 303) and a small N-

terminal lobe (residues 31 to 104), connected via a flexible hinge region, which lies in a cleft between the N-terminal and the C terminal lobes of the complex. The hinge region is the catalytically active site of the complex. The N-terminal lobe comprises of a five-stranded anti-parallel β-sheet and a single α-helix.The C-terminal lobe is composed mainly of α-helices, which contains the substrate-binding site located in a groove on its surface. A glycine-rich loop, which constitutes the consensus kinase sequence Gly-x-Gly-x-x-Gly (residues Gly43-Gly48), and the hinge region (residues Met104-Ser111) surround the catalytically active site of TAK1. Adenosine binds to the TAK1 kinase domain at the interface of the N- and the C-terminal lobes, with the purine base forming two hydrogen bonds with the hinge region. One hydrogen bond is formed with the amide nitrogen atom of Ala107 and the second hydrogen bond is formed with the carbonyl oxygen atom of Glu105 (Brown. K *et al*., 2005). Residues 480-504, which constitute the TAB1 C-terminal region, are sufficent for the binding and activation of TAK1 (sakurani. H *et al*., 2000). Truncation mutation analysis of TAB1 has revealed that a sequence of 30 amino acid residue, α-helical region in TAB1 is indispensable for TAK1 binding under physiological conditions (Ono. K *et al*., 2001). Research studies have also revealed that TAB1 and TAK1 interact via a binding pocket present on the catalytic domain of TAK1. The domain of TAB1 domain that activates TAK1 consists of three regions: an ordered loop spanning from Histidine 468 to Proline 480, a short turn encompassing the region from Tyrosine 481 to Phenylalanine 484 and an α-helix spanning the region from Alanine 485 to Aspartate 494. The α-helix assures the apt arrangement of a motif of aromatic amino acids whose hydrophobicity and morphology closely complements the hydrophobicity and shape of the TAB1 binding pocket in TAK1. The α-helix comprises the sequence, in which the aromatic amino acids are crucial for hydrophobicity. The residues in the α-helix form a ladder in which the side chains of aromatic amino acids clamp into the TAB1 binding region in the kinase domain of TAK1 and mount the hydrophilic residues to the exterior of the helix (Brown. K *et al*., 2005).

Phenylalanine 484 and Tyrosine 488 in TAB1 are crucial for TAK1 binding. These residues are involved in substantial lipophilic associations within the TAB1 binding pocket and hence are essential in stabilizing the TAK1/TAB1 complex (Hartsough. MT *et al*., 1995). The interaction between TAK1 and TAB1 appears to be a locking mechanism.The chimeric protein of TAK1/TAB1 (Ile31-Gln304:His468-Pro405) remains enzymatically active, however, it exhibits no phosphorylation on Thr-184, Thr-187 and Ser-192 of TAK1. Hence the phosphorylation at these sites is not mandatory for TAB1 binding to TAK1. Also the binding of TAB1 appears to be sufficient for activating TAK1 even in the absence of these phosphorylations (Brown. K *et al*., 2005).

**4.3. TAK1 Binding proteins**

The activation of TAK1 protein requires TAK1-binding proteins 1 (TAB1, TAB2, and TAB3 (Yan Ran Xu etal., 2021). TAB1 is an adaptor protein that is constitutively associated with TAK1,even in unstimulated cells through its N-terminal domain, while TAB2 and TAB3 bind to the C-terminus of TAK1 through TAK1-binding domain after stimulation (Shibuya. H *et al*., 1996, Besse. A *et al*., 2007). TAB1 is structurally distinct to TAB2 and TAB3, while as TAB2 and TAB2 abd TAB3 share 48% amino acid sequence with each other.TAB1 mediates autophosphorylation of TAK1 at two threonine rsidues of TAK1 (thr-184, 187 and ser-192). These phosphorylations are dispensable for TAK1 kinase activity (shibuya. H *et al*., 1996, Sakurai.H *et al*., 2000). The global inactivation of TAB1 terminates in embryonic lethality and also leads to defects in development of vital organs like lungs and heart (Komatsu. Y *et al*., 2002) In contrast, several studies proved that TAB2 and its homolog TAB3 play redundant roles in TAK1 activation (Ishitani. T *et al*., 2003). TAB2 and its homolog TAB3 are found to bind ubiquitin to function as an adaptor, tethering TAK1 to the IKK complex.Moreover, TAB2$^{-/-}$ mice have dysregulations in liver development and are embryonically lethal (Shim. JH et al., 2005, Sanjo. H *et al*., 2003). These findings indicate that TAB1 and TAB2 are not functionally

redundant and have discrete roles in regulating TAK1 activity.

There are various considerable evidences which indicate that the requirements for the binding of TAK1binding partners are cell type specific and stimuli specific e.g, the TNF-α induced activation of TAK1 involves a signaling complex with TAB1 and TAB2 in Hela cells (Singhirunnusorn. P *et al*., 2002). In glomerular mesengial cells, TAB1 is necessary for TAK1 activation induced by TGF-β1 (Kim. S. L *et al*.,). Studies in TAB1 null mouse embryonic fibroblasts have revealed that the activation of TAK1 by osmotic stress is TAB1 dependent. However, the activation of TAK1 by IL-1 and TNF-α is independent of TAB1 (Inagaki. M *et al*., 2005). The TAK1-TABs phosphorylation of IKKβ at Ser177 and Ser181, is required for the activation of NF-κB signaling pathway. Furthermore TAK1-TABs complex is also crucial for the MAPK activation. Recent research reports have demonstrated that the TAK1-TABs complex plays important roles in innate immune and inflammatory responses. It is also emerging that this complex controls a large amount of physiological and pathological processes (Yan Ran Xu et al., 2021) Although the TAK1-TABs complex has been widely studied, the roles of the individual binding proteins and the molecular mechanisms responsible for their activation in different cell types remains to be addressed

**4.4. Regulation of TAK1 activity**

Since it is a well known fact, that the activation status of TAK1 is tightly regulated by Association of its binding partners, TAB1, TAB2, and TAB3. Recent evidences indicate the importance of post-translational modifications (PTMs) ofTAK1 and TABs in the regulation of TAK1 activation. Till now, a number of PTMs of TAK1 and TABs have come to light, and these modifications appear to coordinate TAK1 activities depending on the cellular context. Some of the important post translational modifications which play a role in modulating the TAK1-TAB complex formation and TAK1 activation which include: phosphorylation/de-

phosphorylation, methylation, ubiquitination, acetylation, sumoylation and acetyl-glucos-aminylation (O-GlcNAcylation) (Yusuke Hirata *et al*., 2017)

Since the phosphorylation of TAK1 at Thr-187 is crucial for its kinase activity in the TNF receptor (TNFR) signaling, methylation and ubiquitination are also crucial in regulating its activity. Many studies have shown that site-specific phosphorylation and ubiquitination on TAK1 are important in regulating TAK1 activity . Moreover, full activation of TAK1 requires O-linked β-N-acetylglucosaminylation (O-GlcNAcylation) of TAB1 at Ser-395 (Pathak. S *et al*., 2012, Irie. T *et al*., 2000). Once activated, TAK1 auto-phosphorylates four serine (Ser) or threonine (Thr) residues, Thr178, Thr184, Thr187, and Ser192 within the activation loop, (Sakurai. H *et al*., 2002). The phosphorylation of Thr187 and Ser412 is negatively regulated by the phosphatases. TNF-α and IL-1β induce the interaction of TAK1 with dual-specificity phosphatase 14 (DUSP14, also known as MKP6), promoting the de-phosphorylation of TAK1 at Thr187 . The de-phosphorylation of TAK1 at Thr187, is also mediated by PP2A in TGF-β signaling, and PP2Cβ-1, PP2Cε and PP6 in IL-1β signaling (Zhang. H *et al*., 2013 Singhirunnusorn. P *et al*., 2005).

**4.5. TAK1 and Autophagy**

Autophagy is a fundamental and highly conserved cellular process that eliminates molecules and subcellular elements, including nucleic acids, proteins, lipids and organelles, via lysosome-mediated degradation to promote, survival, homeostasis, development and differentiation. During nutrient starvation, autophagy is activated to maintain the supply of essential nutrients to the cell. However, in other circumstances it can serve as a process contributing to cell death known as autophagic cell death (Yahyah *et al*., 2021, Denton. D *et al*., 2012).

The role of TAK1 in the regulation of cellular autophagy has emerged recently. Tak1 promotes cellular autophagy via Tumor necrosis factor-related apoptosis-inducing ligand (TRIAL) The

induction of autophagy by TRIAL is mediated by AMPK.TRAIL activation leads to the activation of TAK1 which in turn activates AMPK: a potent activator of autophagy (Herrero Martin. G *et al*., 2009) TAK-1 has also been known to inhibit S6K1 (by phosphorylation). Under normal conditions (i.e, in the presence of nutrients inside the cell), downregulation of S6K1 phosphorylation (via mTORC1 pathway) is associated with induction of autophagy (cytotoxic autophagy). TAK1 hence, induces autophagy by inhibiting S6K1 phosphorylation (Shin. JH *et al*., 2013)

**4.6. TAK1 and mTOR crosstalk**

mTOR protein is a highly conserved, Ser/Thr kinase, which belongs to PI3K-related kinase family and plays a very important role in the regulation of cell growth and metabolism. It is a 289-kDa protein, and consists of 2549 amino acids consisting of several domain structures which are conserved. mTOR interacts with a variety of proteins to produce two separate multiprotein complexes that are physically and functionally diverse: mTOR complex 1(mTORC1) and mTOR complex 2 (mTORC2) (Guertin and Sabatini, 2007).

Experiments pertaining to immuneprecipitation have revealed that TAK1 binds to S6K1, and carboxy terminal domain trunication of S6K1 abolishes this interaction suggesting that the S6K1 binds to TAK1 via carboxy terminal domain. The binding of TAK1 to S6K1 reduces the phosphorylation of S6K1. Research evidences have also demonstrated that TAK1 interacts with Raptor and co-expression of TAK1and S6K1 triggers a decrease in the binding of S6K1 to raptor. Moreover the binding of S6K1 to raptor increases in raptor dose dependent manner. TAK1 therefore competes with S6K1 for raptor binding and inhibits the phosphorylation and activation of S6K1 (Shin. JH *et al*., 2013). S6K1 has also been found to influence the binding of TAB1 to TAK1, and hence negatively regulates the TLR signaling. S6K1 is also known to interact with TAK1 amino-terminal domain. The amino terminal domain of TAK1 is also responsible for its interaction with TAB1. Also, the TAK1 interaction with TAB1 or S6K1 is

remarkably abolished in presence of TAB1 or S6K1 respectively. These findings clearly demonstrate that the amino terminus of TAK1 is involved in binding to S6K1, and this interaction competes with negatively regulates TAK1 activation by competing with TAK1–TAB1 binding (Kim. SY *et a*l., 2014). By disturbing the interaction between TAK1 and TAB1, S6K1 may also destabilize the AP-1 activating MAP Kinase pathway, as TAB1 is the molecule that is known to recruite p38 MAPK to TAK1 complex (Pathak. S *et al*., 2012, LU. G *et al*., 2006). The interaction between TAK1 and S6K1 thus appears to be vital in TAK1 signaling for both AP-1 activation via JNK pathway and NF-κB activation through the IKK pathway. S6K1 thus, regulates the TLR-2 and TLR-4 mediated signaling pathways in a negative manner (Kim. SY *et al*., 2014). It is also known that the deficiency of S6K1 leads to the enhancement of the kinase activity of TAK1, leading to the increase in the activation of downstream molecules of TAK1 and boosting the NF-κB dependent gene expression upon TLR-2 or TLR-4 stimulation. Deficiency of S6K1 also results in the increased production of pro-inflammatory cytokines including TNF-α IL-1β and IL-6. On the other hand, overexpression of S6K1 represses the production of pro-inflammatory cytokines and also brings down the NF-κB activity (Kim. SY *et a*l., 2014).

**4.7. TAK1 and AMPK**

Since all the physiological and biochemical processes, occurring inside the cell which sustain life requires energy, cells must therefore continually control their energy consumption based on the availability of nutrients and their ATP production potential. Eukaryotes have evolved a sophisticated system that uses the serine/threonine kinase: AMP-activated protein kinase (AMPK) complex to detect low cellular ATP levels. 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a serine/threonine kinase, which plays a key role in regulating energy balance at cellular and whole organism levelsIt is highly conserved in all eukaryotes. In eukaryotes, AMPK is the principal energy sensor and regulator of energy

homeostasis. Energy stress activates it, in response to increased ATP consumption (e.g., exercise, cell proliferation, anabolism) or decreased ATP generation (e.g., low glucose levels, oxidative stress, hypoxia), which is detected as low ATP to AMP and ADP ratios . (Sang-Min Jeon 2016).

In adittion to Liver kinasebB1 and Calmodulin depenent protein kinase kinase 2 (CAMKK2) TAK1 has been suggested as a direct upstream kinase of AMPK. There are various research evidences that advocate the interplay between TAK1 and AMPK, but the interpretation of these evidences have led to conflicting views, therefore the exact role of TAK1 in regulation of AMPK activity is quite ambiguous (Neumann. D 2018). TAK1-TAB1 complex phosphorylates the catalytic subunit of AMPK at thr-172 which was proven by the recombination studies. It is also known that overexpression of TAK1 and TAB1 leads to the enhancement of AMPK phosphorylation in HeLa cells (M. Momcilovic *et al*., 2006). Various forms of stress like hypoxia and osmotic schock, which act as the activators of AMPK are also known to activate TAK1 (Fuji. N *et al*.,2006). The fact that the activators of TAK1 like TNF-α, TGF-β and IL-1 also have stimulatory effects on AMPK, hints at the interaction between the two kinases 134. Knockdown of TAK1 in mouse embryonic fibroblasts abates the cells abilty to be activated in response to metformin, AICAR and oligomycin.

## 5. Identification of Research Gaps

Although autophagy is usually thought to be a favourable mechanism that improves cell survival by inhibiting growth, it has been vaguely attributed to enhance growth. High levels of autophagy have also been linked to the promotion of cell death. As a result, it's critical to identify signalling checkpoints that keep autophagy from reaching dangerous levels while also allowing the cell to tolerate prolonged starvation. While nutrition recycling during autophagy has the potential to operate as an intrinsic negative feedback, the cell must have dedicated mechanisms to prevent this from happening.

To answer these concerns, it is important to understand how the signals that control nutrition and energy dynamics work together to preserve homeostasis. It's also crucial to comprehend how the two seemingly opposed signals must interact.long-term growth during cancer genesis.

In this context, this study will be done to evaluate a potential cross talk between mTORC1 dependent nutrition dynamics and TAK1 regulated energy homeostasis.

We will use a combination of genetic and biochemical approaches like mutational analysis of TAK1 and TAK1 Knock out cell line to monitor TAK1/mTORC1 crosstalk.

## 6. Methodology

### 6.1. Cloning of TAK1 sgRNA in pSpCas9(BB)-2A-Puro (PX459) knockout plasmid

The gRNA cloning protocol consists of five overall steps:

- Annealing & 5' phosphorylation of the oligonucleotides.
- Digestion of the vector backbone, CIP treatment and Gel purification of digested vector.
- Ligating annealed oligonucleotides into the digested plasmid.
- Preparation of DH5 alpha competent cells for transformation of ligated vector.
- Confirmation of positive knockout clones through PCR.

### 6.2. Cell Culture and treatment

HEK 293-T cells (human embryonic kidney cells supplemented with simvian *"T-antigen"*) will be maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml penicillin and 100 μg/ml streptomycin at 37°C in an incubator with 5% CO2 concentration. Cells will be routinely passaged twice a week. Cells will be treated with rapamycin (50nM) for 15–20 min before serum stimulation for 30 min and then Cells will be further examined, according to the experimental needs.

### 6.3. Transfections

Transfections of TAK1, S6K and AMPK plasmid DNAs will be carried out using PEI (Polyethylene amine) as the transfection reagent. HEK-293 cells will be grown to 100% confluency in T75 flasks. The cells will be then trypsinised and seeded in 6cm plates. The cells will be grown to 70% confluence prior to transfection, by growing them overnight in the humidified incubator (5% $CO_2$, 95% air and 37$^{o}$C). Transfection mixture will be prepared by adding 3µg of DNA to 400µL of serum and antibiotic free media. The mixture will then be allowed to stand for 5 minutes at room temperature. 15µL of PEI (1µg/mL) will be added to the mixture and the mixture allowed to stand for 20 minutes. Fresh and complete media will be added to the cells. After changing the media of the cells, the transfection mixture will be added and the cells placed in the humidified incubator.

### 6.4. Isolation and selection of TAK-1 Knockout cells

Prior to selection of transfected cells, Puromycin kill Curve assay will be done to calculate the lethal concentration of Puromycin by adding different concentrations ranging from (0.4ug/ml-3ug/ml) of Puromycin to cells. The no. of live cells will be calculated using Trypan Blue Dye and hemocytometer. The amount of puromycin just below the lethal concentration (1ug/ml) will be used for selection.

### 6.5. Selection of Transfected cells

After successful incubation of 60 hours post-transfection, puromycin treatment will be started.Puromycin treatment will be given over a period of time, till the control (mock) will be cleared up for any live cells. After total cell death in control, puromycin treatment will be further given to 2 more days. Puromycin treatment will be stopped and the remaining cells will be picked and allowed to grow again.

**6.6. Immuno-precipitations and Western blotting**

48 hrs, post transfection, cells will be harvested, using ice-cold PBS.. Lysis will be done on ice in a buffer containing 50 mM Tris–Cl (pH 7.5), 10 mM MgCl2, 5 mM EDTA, 2 mM DTT, 50 Mm β-Glycero-phosphate, 0.05% Triton X-100 and Protease inhibitor cocktail(Sigma) for 30 min. Precipitated material will be removed by centrifugation (microcentrifuge, 13,000 rpm for 15 min) and cleared lysates will be incubated overnight with appropriate antibody immobilized on protein G-Agarose beads. Beads will be washed thrice with lysis buffer containing 500 mM NaCl following a final wash with salt free lysis buffer to remove the salt. Laemmli sample buffer will be added to the beads, which will be then subjected to SDS-PAGE. Proteins will be transferred on PVDF membrane and will be probed with different antibodies at indicated concentrations and will be analysed using Odyssey infrared imager (LI-COR).

**6.7. Immunofluorescence confocal microscopy.**

For immunofluorescence confocal microscopy, HEK-293T cells will be plated onto coverslips in 35mm petri dishes. Cells will be transfected with plasmids as reqd. 48 hrs post transfection Cells will be incubated in full media or EBSS for 6hr or treated with 100uM chloroquine for 1h. Post- treatment the medium from the cells will be aspirated and washed twice with 1X PBS at room temperature followed by fixation in 4% paraformaldehyde for 5 min. at room temperature or with 100% methanol for 5 min at −20°C. Coverslips will be mounted using ProLong Gold Antifade Mountant (Invitrogen) and analyzed by confocal microscopy using the Carl Zeiss Scanning Microscope.

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